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EPP - Standard Operating Procedure

(only for selected experiments intended to transfer results from one lab to the other)

Title: Performing a batch and chemostat cultivation with *P. putida* in lab-scale

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Instruction

Performing a batch and chemostat cultivation with *P. putida* in lab-scale

1 *Introduction / Purpose*

This SOP is a standardized protocol for performing chemostat cultivations with *Pseudomonas putida* in a lab scale bioreactor. Chemostat cultures are performed to determine specific growth parameters or investigate physiological characteristics of bacterial strains. Therefore a bespoke fermentation procedure for *P putida* is performed according to Vallon et al. (2015).

2 *Equipment and chemicals*

2.1 Equipment

- ⇨ 500 ml baffled shake flask
- ⇨ 1000 ml baffled shake flask
- ⇨ 3 L lab scale bioreactor from Bioengineering
- ⇨ Process control equipment for an automated pH-, T and pressure regulation
- ⇨ Peristaltic pumps for addition of base, feed medium and harvest
- ⇨ If available, exhaust gas analysis

2.2 Chemicals

Refer to 3. Media and buffers

2.3 Bacterial strains

Pseudomonas putida KT2440

3 *Media and buffers*

3.1 M12 medium

To perform a chemostat cultivation with *P. putida* the minimal medium M12 is used. Following table shows the composition of M12 medium which is used for preculture and cultivation in bioreactor (Vallon et al., 2015). Additionally you need to add further components according to the type of medium (see Table 2).

Table 1 Composition of M12 medium for preculture, batch and chemostat cultivation

Component	Concentration [g/l]
$MgSO_4$ x $7H_2O$	0.40
$CaCl_2 \times 2H_2O$	0.04
$(NH_4)_2SO_4$	2.20
NaCl	0.02

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KH_2PO_4	2.000
$ZnSO_4$ x $7H_2O$	0.002
$MnCl_2$ x $4H_2O$	0.001
Na_3-Citrate x $2H_2O$	0.015
$CuSO_4$ x $5H_2O$	0.001
$NiCl_2$ x $6H_2O$	2.00E-05
$NaMoO_4$ x $2H_2O$	0.00003
H_3BO_3	0.0003
$FeSO_4$ x $7H_2O$	0.01

Table 2 Additional components for the medium

Component	Preculture 1	Preculture 2	Batch	Feed
$C_6H_{12}O_6$	4 g/L	4 g/L	15 g/L	15 g/L
Yeast extract (YE)	0.5 g/l	-	-	-
MOPS	10 g/l	10 g/l	-	-

Before you inoculate the preculture, adjust the pH to 7 by titration of 5 M KOH solution. For titration of the biosuspension in the bioreactor use 25% NH_4OH or NaOH solution.

4 Procedures

4.1 Seed-train for preculture

For a successful growth of the bacteria, it is necessary to start with a preculture in shaking flask before inoculating the bioreactor. Therefore follow this procedure:

1. Prepare the media according to Table 1 and Table 2
2. Preculture 1: Inoculate a 500 ml baffled shaking flask containing 50 ml M12 Medium (4 $g_{glucose}$/l; 0.5 g_{YE}/, pH 7) from your prepared glycerol stock and incubate it at 30 °C, 140 rpm until OD ~2.
3. Preculture 2: Prepare a 1000 ml baffled shaking flask containing 100 ml M12 (4 $g_{glucose}$/l, pH 7). Use the first preculture at OD~2 to inoculate the second preculture at OD_0 = 0.003. Incubate the cells at 30 °C, 140 rpm until OD ~3.
4. Use the 100 ml of preculture 2 to inoculate the bioreactor. Always work aseptic

4.2 Preparation of bioreactor and batch phase

Before you start the fermentation, prepare your bioreactor for cultivation conditions. First, sterilize the bioreactor and ensure a monoseptic operation during the process. Fill in the batch medium and consider the additional volume of the inoculum. Set the optimal process conditions listed in Table 3

Table 3 Optimal process parameters for cultivation of P. putida in a lab-scale bioreactor

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Parameter	Value
Aeration [l/min]	1 v/(V_R*min)
Temp. [°C]	30
pO_2 [%]	> 20
Overpressure [bar]	0.5
pH	7

Start the batch process by inoculation of the bioreactor using the preculture 2, reaching a start-OD of ~ 0.2. In best case use automated pH- and pO_2- control. The pO_2 should be kept above 20 % during the fermentation by adjusting stirrer speed. After the initial glucose is consumed switch to chemostat operation by starting a constant feed and harvest.

4.3 Chemostat cultivation under carbon limitation

For chemostat cultivation you will need two peristaltic pumps for feed and harvest. The feed rate F is characterized by the reactor volume V_R and the dilution rate D (equals µ):

$$F = D * V_R$$

To reach a steady state in the chemostat culture it takes approx.. 3 – 5 residence times τ

$$\tau = \frac{1}{D}$$

Prepare the amount of feed to supply enough volume for 5 τ. The continuous harvest can be realized by using the signal of a balance on which the bioreactor is placed (gravimetric method). After the glucose in the batch phase is metabolized the chemostat can be run by starting the continuous feed and harvest. Also make sure that the stirrer speed is set to a constant rate for maintaining pO_2 above 40 %. If available use a syringe pump to feed antifoam at a rate of 100 µl/h. After reaching a steady state characterized by $dc_X/dt = 0$ and $dc_S/dt = 0$ (at least after 3 × τ), cells should grow at a constant growth rate. Now you can take samples for your desired kind of investigation.

5 remarks/troubleshooting:

6 Biosafety

Cells must be handled according to S1 lab safety.

Publication bibliography

Vallon, Tobias; Simon, Oliver; Rendgen-Heugle, Beate; Frana, Sabine; Mückschel, Björn; Broicher, Alexander et al. (2015): Applying systems biology tools to study n -butanol degradation in Pseudomonas putida KT2440. In *Eng. Life Sci.* 15 (8), pp. 760–771. DOI: 10.1002/elsc.201400051.

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